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The RanGAP1-RanBP2 Complex Is Essential for Microtubule-Kinetochore Interactions In Vivo

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Summary

RanGAP1 is the activating protein for the Ran GTPase. Vertebrate RanGAP1 is conjugated to a small ubiquitin-like protein, SUMO-1 [1, 2]. This modification promotes association of RanGAP1 with the interphase nuclear pore complex (NPC) through binding to the nucleoporin RanBP2, also known as Nup358. During mitosis, RanGAP1 is concentrated at kinetochores in a microtubule- (MT) and SUMO-1-dependent fashion [3]. RanBP2 is also abundantly found on kinetochores in mitosis [3]. Here we show that ablation of proteins required for MT-kinetochore attachment (Hec1/Ndc80, Nuf2 [4–6]) disrupts RanGAP1 and RanBP2 targeting to kinetochores. No similar disruption was observed after ablation of proteins nonessential for MT-kinetochore interactions (CENP-I, Bub1, CENP-E [7–9]). Acquisition of RanGAP1 and RanBP2 by kinetochores is temporally correlated in untreated cells with MT attachment. These patterns of accumulation suggest a loading mechanism wherein the RanGAP1-RanBP2 complex may be transferred along the MT onto the kinetochore. Depletion of RanBP2 caused mislocalization of RanGAP1, Mad1, Mad2, CENP-E, and CENP-F, as well as loss of cold-stable kinetochore-MT interactions and accumulation of mitotic cells with multipolar spindles and unaligned chromosomes. Taken together, our observations indicate that RanBP2 and RanGAP1 are targeted as a single complex that is both regulated by and essential for stable kinetochore-MT association.

Results and Discussion

We previously reported that RanGAP1 associates with kinetochores in a SUMO-1- and MT-dependent fashion [3]. Several lines of evidence indicated that this targeting occurs in association with RanBP2. First, these proteins remain tightly bound throughout the cell cycle. Anti-RanBP2 antibodies precipitated SUMO-1-conjugated RanGAP1 with equal efficiency from interphase and mitotic HeLa cell extracts (Figure S1A). Equal coprecipitation efficiencies were also observed between interphase and mitotic *Xenopus* egg extracts (data not shown). Second, RanGAP1 and RanBP2 colocalize during mitosis [3]. Third, RanGAP1 spindle localization is absolutely dependent upon RanBP2 (Figure S1B). Moreover, RanBP2 and RanGAP1 recruitment to kinetochores showed identical requirements for other kinetochore components and identical timing during unperturbed cell cycles (see below). These results provide strong support for our earlier speculation that RanGAP1 and RanBP2 are targeted as a complex during mitosis [3]. For the purposes of this report we have thus assumed that RanGAP1 and RanBP2 are localized on spindles as a single entity.

We have investigated how and why the RanGAP1-RanBP2 complex is targeted to kinetochores. We initially examined which features of the kinetochore are critical for recruitment of RanGAP1 and RanBP2. Because their kinetochore localization was MT dependent, we utilized RNAi to suppress the expression of kinetochore proteins that are required for stable MT-kinetochore interactions (Hec1/Ndc80 and Nuf2 [4–6]) and analyzed the effect on RanGAP1 and RanBP2 targeting. We also examined the localization of RanGAP1 and RanBP2 after the depletion of CENP-E. CENP-E loss does not abolish MT attachment but does cause decreased numbers of MT-kinetochore attachments and loss of tension on kinetochores [9, 10]. Finally, we examined the fate of RanGAP1 and RanBP2 after depletion of CENP-I and Bub1 [7, 8], kinetochore proteins that are implicated in other aspects of kinetochore function and in spindle checkpoint signaling.

Depletion of Hec1 and Nuf2 by RNAi compromised kinetochore-MT attachment in mitotic cells and thus impaired chromosome alignment (Figure 1) [6, 11]. Under these circumstances, RanGAP1 and RanBP2 were no longer associated with kinetochores, showing that Hec1 and Nuf2 are essential for targeting both proteins (Figure 1, data not shown). While Hec1 and Nuf2 are required for recruitment of the RanGAP1-RanBP2 complex, they are not sufficient: Hec1 and Nuf2 are retained on kinetochores in nocodazole-treated cells ([5]; data not shown), although RanGAP1 and RanBP2 are not [3]. This fact suggests either that Hec1 and Nuf2 are not competent to recruit the RanGAP1-RanBP2 complex prior to MT-kinetochore attachment or that they are required indirectly through their role in stabilizing MT interactions. We favor the latter alternative, since there is no precedent for direct interaction of Hec1 or Nuf2 with the RanGAP1-RanBP2 complex. Consistent with this notion, RNAi-mediated depletion of CENP-I and Bub1, which are not essential for MT-kinetochore attachment [7, 8], had no effect on the kinetochore binding of either RanGAP1 (Figure S2A) or RanBP2 (data not shown).

As reported earlier [9, 10], depletion of CENP-E resulted in mitotic arrest with a mixture of aligned and unaligned chromosomes. In CENP-E-depleted cells,

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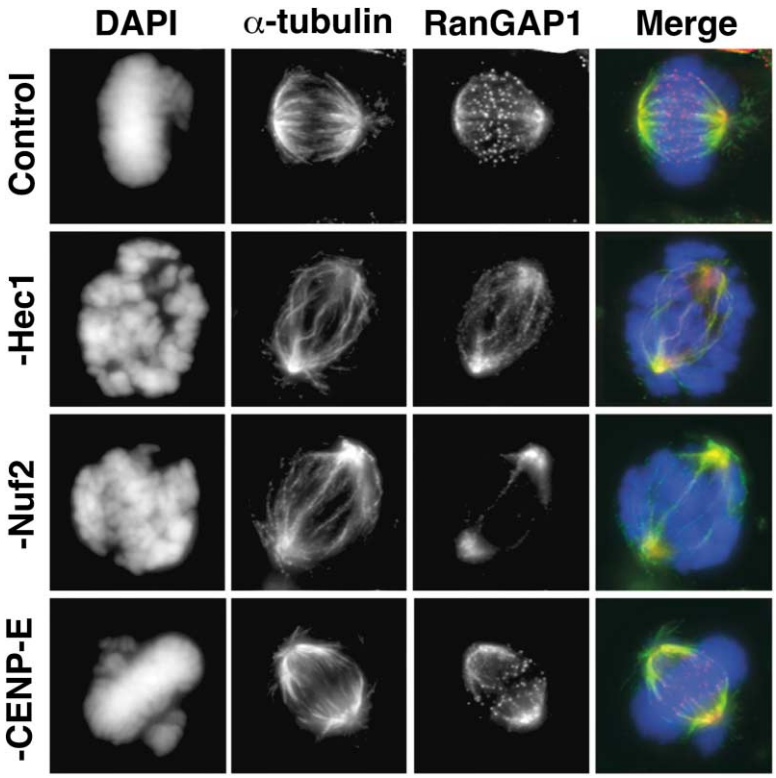

Figure 1. Hec1 and Nuf2 Are Required for RanGAP1-RanBP2 Complex Localization

HeLa cells were transfected with siRNA oligonucleotides specific for Hec1, Nuf2, and CENP-E for 24–48 hr to knockdown the expression of indicated proteins. Depletion of Hec1, Nuf2, and CENP-E could be achieved after 48 hr, as evidenced by the undetectable levels of these proteins at kinetochores in cognate RNAi-treated cells as compared to control RNAi-treated cells (data not shown). Cells were fixed as described in the Supplemental Experimental Procedures. Kinetochore localization of RanGAP1 (red) was examined by using specific antibodies. The microtubules were visualized with anti-α-tubulin (green) and DNA with DAPI (blue).

RanGAP1 and RanBP2 were found at kinetochores of aligned chromosomes, whereas kinetochores of unattached chromosomes lacked detectable staining (Figure 1, data not shown). In the absence of CENP-E function, MT-kinetochore attachment is achieved, although the number of kinetochore MTs is decreased and tension on kinetochores is compromised [9]. Notably, neither RanGAP1 nor RanBP2 was displaced as a result of decreased kinetochore tension after loss of CENP-E. Consistent with this observation, RanGAP1 and RanBP2 localized to kinetochores in taxol-treated cells (data not shown). Together, these data indicate that RanGAP1 and RanBP2 accumulation at kinetochores is compromised in the absence of MT attachment but does not appear to be sensitive to loss of kinetochore tension.

We further examined the correlation between MT attachment and RanGAP1-RanBP2 complex acquisition by kinetochores under unperturbed conditions through careful comparison of Mad1 and RanGAP1 localization in untreated HeLa cells. Mad1 and Mad2 are checkpoint proteins that become abundantly associated with unattached kinetochores in close correlation with spindle checkpoint activation [12]. As reported earlier [13], Mad1 (Figure 2A) and Mad2 (data not shown) localized on the nucleoplasmic side of the NPC during interphase; RanGAP1 (Figure 2A) and RanBP2 (data not shown) associated with the cytoplasmic side of the NPC [14]. During nuclear envelope (NE) breakdown in prophase, Mad1 prominently localized to kinetochores, although residual Mad1 could still be observed on the NE (Figure 2B). RanGAP1 (Figure 2B) and RanBP2 (data not shown) were still found at the NE at this stage, but not on kinetochores. After NE breakdown was complete, the RanGAP1-RanBP2 complex did not accumulate on kinetochores prior to MT attachment. This could be clearly seen through the mutually exclusive localization of RanGAP1 and Mad1 (Figure 2C) [12]. Mad1 localized to both kinetochores on a fully unattached chromosome, while RanGAP1 bound neither (Figure 2C, insert 1). The mutually exclusive localization of RanGAP1 and Mad1 was even more apparent in the case of a chromosome that had a single MT-attached sister kinetochore (Figure 2C, insert 2): Mad1 was found only on the unattached sister kinetochore, whereas RanGAP1 was found only on the attached sister. Like Mad1, Mad2 showed a distribution pattern that was inverse to the RanGAP1 pattern (data not shown). Furthermore, RanBP2 deposition on kinetochores was also mutually exclusive to both Mad1 and Mad2 accumulation (data not shown).

These data support the idea that RanGAP1-RanBP2 complex accretion on kinetochores is closely coupled to MT attachment under unperturbed conditions. It is plausible that MT attachment causes structural changes at the kinetochore that permit stable association of the RanGAP1-RanBP2 complex. Another attractive possibility that is not mutually exclusive with structural changes at kinetochores is that the RanGAP1-RanBP2 complex binds to MT and is transferred from the MT to the kinetochore. A similar loading model has previously been proposed for the DASH complex in budding yeast, which accumulates on maturing kinetochores in a MT-dependent fashion [15]. It is notable that the complementary kinetochore localizations of proteins from the nucleoplasmic (Mad1 and Mad2) and cytoplasmic (RanGAP1 and RanBP2) faces of the NPC are oppositely determined by MT attachment. Interestingly, the Nup107-

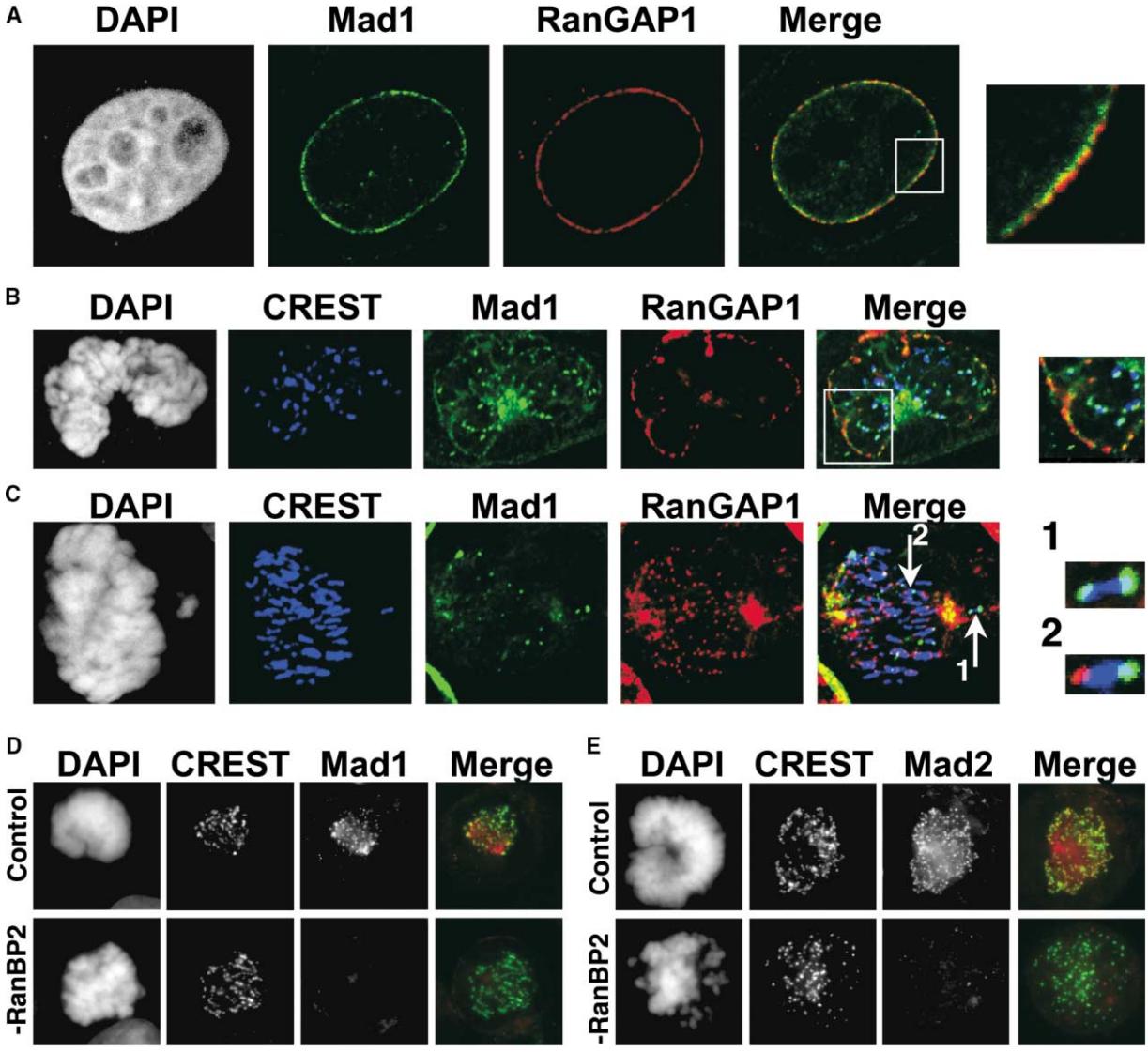

Figure 2. Mad1 and Mad2 Require the RanGAP1-RanBP2 Complex for Kinetochore Binding but Do Not Colocalize on Unattached Kinetochores

HeLa cells (A–C) or RGG cells (D and E) were permeabilized and fixed as described in the Supplemental Experimental Procedures. The cells in (A), (B), and (C) were stained for Mad1 (green) and RanGAP1 (red) with specific antibodies and fluorescent secondary antibodies. Where indicated, immunofluorescent staining with CREST sera is shown in blue to show the localization of mitotic centromeres. The cells in (D) and (E) were stained for CREST (Green) and Mad1 or Mad 2 (Red), as indicated.

(A) During interphase, RanGAP1 is localized on the cytoplasmic face of the NPC, while Mad1 staining is on the nucleoplasmic face of the NPC.

(B) During prophase, Mad1 is recruited to loci adjacent to centromeres prior to the release of RanGAP1 from the NPC.

(C) During metaphase, RanGAP1 and Mad1 show inverse staining patterns. Insert 1 shows a chromosome where both kinetochores are unattached; note the lack of RanGAP1 staining. Insert 2 shows a chromosome where a single kinetochore has become attached. Note that this kinetochore has both released Mad1 and acquired RanGAP1, while its sister remains associated exclusively with Mad1.

(D and E) RGG cells were transfected with RanBP2 siRNA oligonucleotides and examined by immunflourescence 86 hr after transfection. Note the absence of Mad1 and Mad2 recruitment during prophase to unattached kinetochores.

160 subcomplex of NPC proteins has been shown to reside on both sides of the pore during interphase [16], and it is bound to kinetochores in a MT-independent fashion from prophase to late anaphase [16]. It is attractive to speculate that the Nup107-160 subcomplex might play an important role in mitotic recruitment of proteins from both nuclear and cytoplasmic sides of the NPC to kinetochores. If this were the case, biochemical and/or structural changes in the Nup107-160 subcomplex in response to MT attachment may have some role in determining how other NPC components accumulate on the kinetochore.

To determine the significance of mitotic RanGAP1-RanBP2 complex targeting, we depleted RanBP2 by using RNAi in RGG cells, a stable, HeLa-derived cell line expressing a green fluorescent protein-labeled chimeric protein consisting of HIV-1 Rev and a hormone-inducible nuclear localization sequence (Rev-GR-GFP [17]). West-

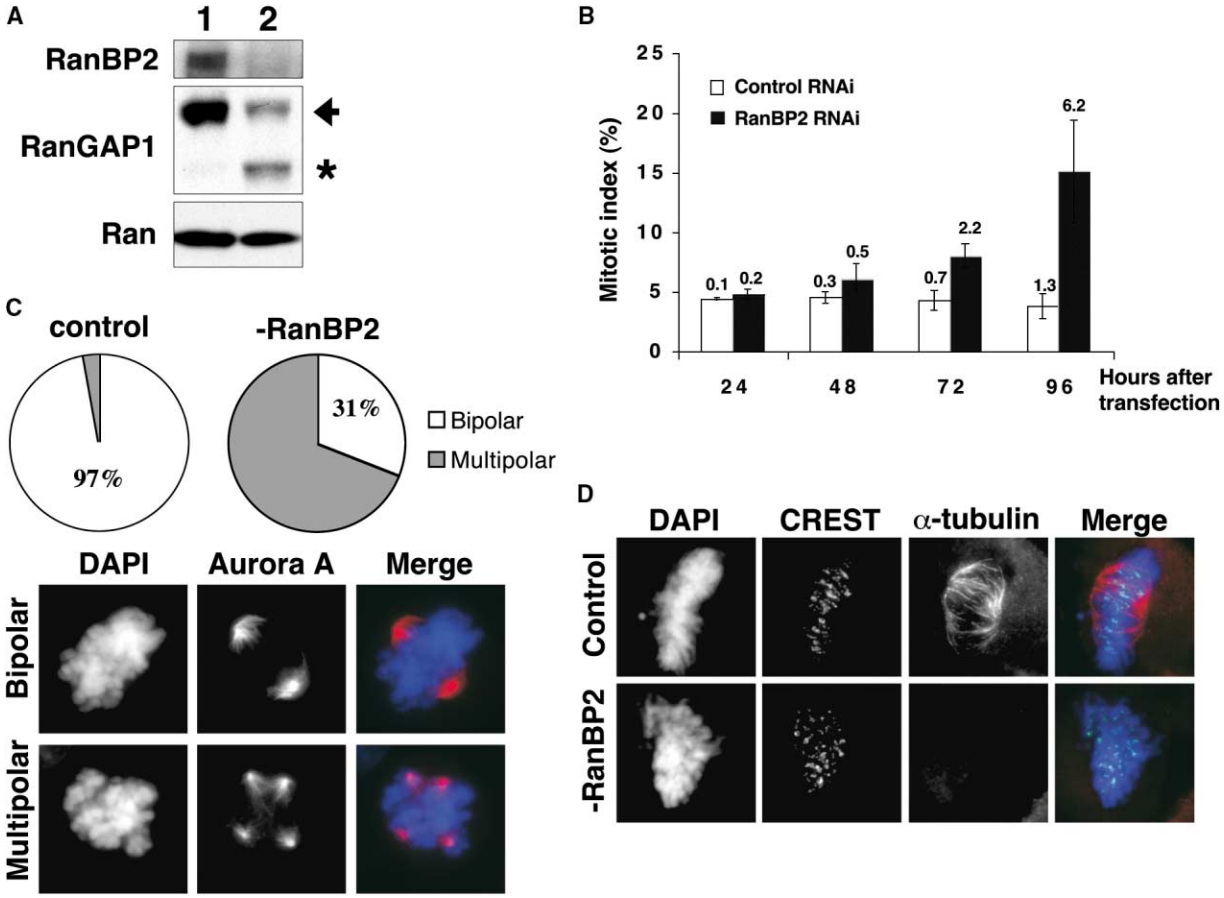

Figure 3. RanBP2 Depletion in RGG Cells by RNAi Causes Mitotic Arrest with Defective Spindle Assembly

RGG cells were transfected with siRNA oligonucleotides specific for RanBP2 or control oligonucleotides and analyzed after 86 hr unless otherwise indicated. The relatively long time required for depletion of RanBP2 may reflect a slow turnover rate for this protein.

(A) Total cell lysates were prepared from control (1) or RanBP2-depleted (2) cells. The lysates were subjected to SDS-PAGE and immunoblotted for RanBP2, RanGAP1, and Ran by using specific antibodies. Ran blot serves as a loading control. The arrow indicates SUMO-1 conjugated form of RanGAP1, while the asterisk represents unconjugated form of RanGAP1. We reproducibly observed that a substantial fraction of RanGAP1 became deconjugated from SUMO-1 in these samples, consistent with earlier reports indicating that RanBP2 protects SUMO-conjugated RanGAP1 from deconjugation by isopeptidases [28].

(B) Mitotic index was calculated at different intervals after control or RanBP2 siRNA transfection by counting immunopositive cells for MPM2 and phospho-H3 antibodies over total DAPI-positive cells. The numbers above bars indicate percentage of cells dead over total cells counted. In our hands, mitotic accumulation after RanBP2 depletion was more apparent in RGG cells than in other cell lines that we tested (e.g., HeLa, U2OS).

(C) Control and RanBP2-depleted cells were fixed with formaldehyde and analyzed. Pie charts show the percentage of mitotic cells with bi- and multipolar spindles. Lower panel shows immunofluorescence micrographs of bi- and multipolar spindles in RanBP2-depleted cells. The spindle poles are stained for Aurora A (red) by using antibodies and DNA with DAPI (blue).

(D) To examine kinetochore MT stability, control and RanBP2-depleted cells were subjected to cold treatment for 10 min before fixing with 4% paraformaldehyde. Cells were then stained for microtubules (red), centromeres (green), and DNA (blue) by using α-tubulin antibody, CREST antiserum, and DAPI, respectively.

ern blotting showed that RanBP2 levels were significantly decreased (>80% depletion compared to controls) 86 hr after transfection of siRNA oligonucleotides (Figure 3A). Although RanBP2-depleted cells still localized other nucleoporins to interphase NPCs (Figure S3A; see also [18]), RanGAP1 did not associate with the NE in the absence of RanBP2 (Figure S3B), consistent with earlier data indicating that RanBP2 binding is critical for its interphase targeting [19]. RanBP2-depleted RGG cells showed an increased mitotic index after 48 hr (Figure 3B), with over 15% of the cells accumulating in mitosis 96 hr after transfection. When chromosome distribution and spindle assembly were examined, RanBP2-depleted cells showed aberrant MT structures and an obvious failure of chromosome alignment on the metaphase plate (Figure S3, see also [18]). Simultaneous depletion of RanBP2 and Mad2 by RNAi reverted the elevation of mitotic index but also caused a dramatic increase in cells with micronuclei (data not shown), possibly reflecting inappropriate exit from mitosis without accurate chromosome segregation. These results indicate that RanBP2-depleted cells arrest in mitosis through activation of the mitotic spindle assembly checkpoint.

Although most spindles were multipolar after RanBP2 depletion, there was a striking similarity among the bipolar spindles formed in RanBP2-, Hec1-, and Nuf2-depleted RGG cells (Figures 1 and S1). Under all three conditions, spindles were longer than those of control cells with unaligned chromosomes: cells transfected with control oligonucleotides showed an average interpolar distance of 12.1 $\pm$ 1.2 μm (n = 25) prior to chromosome alignment, whereas RanBP2-depleted mitotic cells with bipolar spindles had an average interpolar distance of 16.6 $\pm$ 1.5 μm (n = 25), which was closer to Hec1-depleted cells (16.1 $\pm$ 2.0 μm) and Nuf2-depeleted cells (16.4 $\pm$ 1.8 μm). This similarity prompted us to examine the stability of kinetochore-MT attachment in RanBP2-depleted cells. As described previously for cells depleted of Nuf2 [6], we examined whether kinetochore MTs were sensitive to cold [20]. We subjected RanBP2-depleted cells to cold treatment for 10 min prior to fixation. The cells were stained with anti-α-tubulin antibodies and with CREST autoimmune sera that recognize centromeric proteins [21] (Figure 3D). While the control cells showed clear arrays of cold-stable kinetochore MTs, few kinetochore MTs were visible in the RanBP2-depleted cells. This observation suggests that a failure to form stable MT-kinetochore interactions may contribute to spindle defects in RanBP2-depleted cells. These findings are largely consistent with a recent report from Salinas et al. [18], who concluded that RanBP2 depletion causes kinetochore defects, resulting in a spindle checkpoint-dependent mitotic arrest. Since RanBP2 becomes mislocalized in mitotic cells after RNAi-mediated depletion of Hec1 and Nuf2 (Figure 2), our findings also suggest that some part of the failure to form stable MT-kinetochore interactions in the absence of Hec1 and Nuf2 [5, 6] may be related to an inability to correctly recruit the RanGAP1-RanBP2 complex under these circumstances.

We examined the localization of a number of kinetochore components after RanBP2 depletion. Kinetochores of RanBP2-depleted cells lacked Mad1, Mad2, CENP-E, and CENP-F, a kinetochore-associated passenger protein that interacts with CENP-E (Figure 2A, Figure S2). Mislocalization of these proteins was remarkable for two reasons: First, RanGAP1 and RanBP2 are not associated with kinetochores when Mad1 and Mad2 are present (Figure 2C), seemingly precluding direct binding of Mad1 and Mad2 to the kinetochores through the RanGAP1-RanBP2 complex. Our findings may thus imply an indirect requirement for the RanGAP1-RanBP2 complex in loading of Mad1 and Mad2 onto kinetochores. Changes in Ran-GTP levels may contribute to this phenotype, since recent experiments in *Xenopus* egg extracts demonstrated that Mad1 and Mad2 are released from kinetochores by elevated Ran-GTP concentrations [22]. Second, the accumulation of checkpoint proteins on kinetochores is typically closely coupled to activation of spindle checkpoint arrest pathways [12]. However, RanBP2-depleted cells showed a strong Mad2-dependent checkpoint arrest in mitosis without such accumulation (data not shown, see also [18]). Spindle checkpoint arrest of RanBP2-depleted cells in the absence of kinetochore accumulation of multiple checkpoint components is thus unusual, although not unprecedented [11]. Particularly, this phenotype is again reminiscent of the defects observed in Hec1-depleted cells, which fail to accumulate detectable levels of Mad1 and Mad2 at their kinetochores yet mitotically arrest in a Mad2-dependent fashion [11].

Our findings are largely consistent with those of Salinas et al. [18], who observed that CENP-F, dynein and checkpoint components (CENP-E, Mad1 and Mad2, and Zw10), fail to bind kinetochores in the absence of RanBP2. Through additional electron microscopy studies that showed altered kinetochore morphology, they concluded that RanBP2 depletion extensively or completely disrupts kinetochore formation. Surprisingly, our further analysis showed that three proteins associated with the kinetochore throughout mitosis (Hec1, Nuf2, and CENP-I [5, 7, 23]) retained their correct localization in RanBP2-depleted cells (Figure S2), arguing that many of the underlying kinetochore structures still assemble in the absence of RanBP2. Moreover, checkpoint components Bub1 and BubR1 also remained on kinetochores (not less than 85% of levels in control cells; Figure S2), arguing that many aspects of the cell cycle regulatory machinery remain intact in the absence of RanBP2.

RanBP2-depleted RGG cells revealed a high frequency of multipolar spindles among the mitotically arrested population (Figure 3C). We assessed the number of spindle poles by Aurora-A staining [24] in control and RanBP2 siRNA-treated cells to determine the proportion of cells that were multipolar: while 3% of mitotic RGG cells transfected with the control oligonucleotide formed multipolar spindles, 69% of the cells transfected with the oligonucleotide directed against RanBP2 were multipolar. These observations were interesting in light of previous reports that overexpression of the Ran-GTP binding protein RanBP1 leads to unscheduled centrosome splitting [25, 26].

To determine whether the additional poles contained centrosomes, RanBP2-depleted cells were stained with antibodies against human Centrin [27]. The number and distribution pattern of centrosomes both during interphase (data not shown) and mitosis (Figure 4) differed significantly compared to control RNAi-treated cells: more than 95 percent of the control mitotic cells possessed two centrosomes, each of which contained a pair of centrioles. These centrosomes were distributed to opposite spindle poles. The majority of multipolar cells in the RanBP2-depleted samples (72%) also possessed two pairs of centrioles. These centrioles were typically found within MT organizing centers (MTOCs) at spindle poles, although not all MTOCs had foci of Centrin staining. Spindles possessing single, unpaired centrioles within their MTOCs were also evident in RanBP2-depleted cells (17%). Some MTOCs within these cells lacked Centrin foci, indicating that they did not possess centrioles. Finally, around 11% of the multipolar RanBP2-depleted cells had more than two pairs of centrioles, not all of which were associated with obvious MTOCs. It is unclear whether the maldistribution of centrioles in RanBP2-depleted cells is a primary result of RanBP2 disruption or a secondary phenotype, caused indirectly by defects during earlier mitotic divisions in the absence of adequate levels of RanBP2.

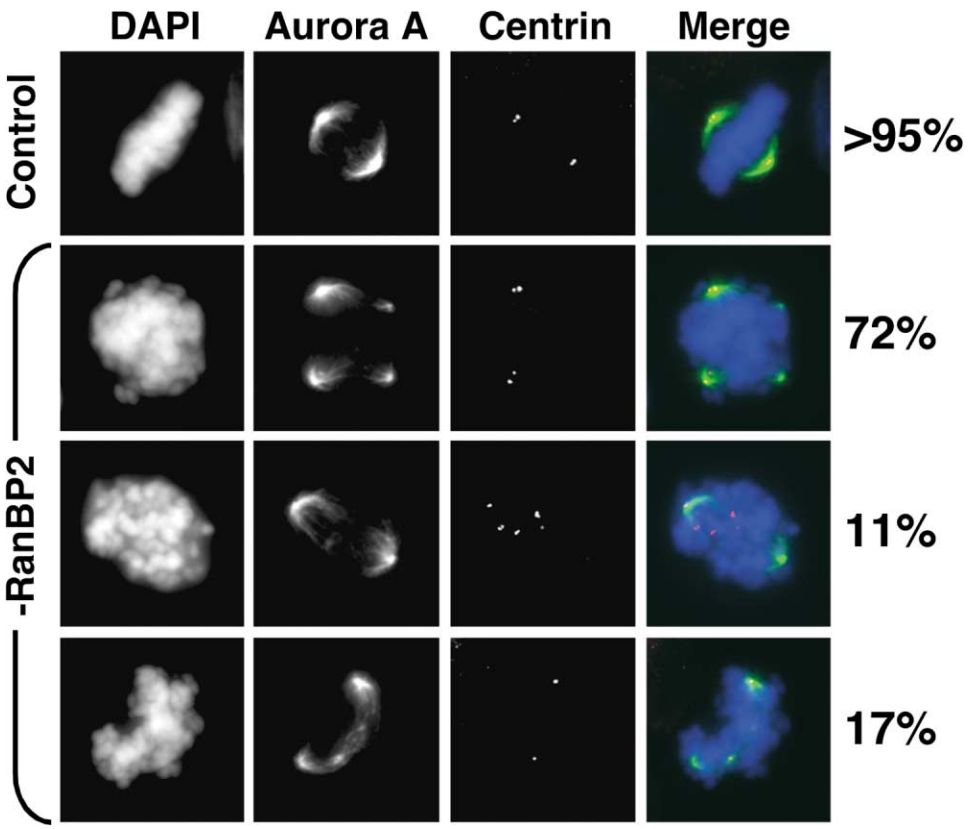

Figure 4. Disruption of Spindle Poles in RanBP2-Depleted Cells

RGG cells were transfected with siRNA oligonucleotides specific for RanBP2 or control oligonucleotides and processed for immunostaining after 86 hr. Cells were stained with Aurora A (green) and Centrin (red) antibodies for visualizing spindle poles and centrosomes, respectively. DNA was visualized by DAPI staining. Although over 95% of the control mitotic cells formed bipolar spindles where each pole contained a single pair of centrioles, the number of poles and centrioles in RanBP2-depleted cells varied widely. Numbers to the right of the RanBP2 depleted cells (−RanBP2) show the percentage of multipolar cells found in each of the major phenotypic classes (see text for further details).

However, the appearance of acentrosomal MTOCs in the majority of RanBP2-depleted cells clearly indicates that the RanGAP1-RanBP2 complex plays direct or indirect roles in spindle assembly beyond its function in kinetochore-MT attachment.

In summary, our results indicate that MT attachment is a critical event for recruitment of the RanGAP1-RanBP2 complex to kinetochores. The mitotic phenotypes of RanBP2- and Hec1- or Nuf2-depleted cells are very similar, possibly suggesting that RanBP2 recruitment may be an important aspect of Hec1 and Nuf2 function. The RanGAP1-RanBP2 complex plays an important role at the kinetochore by stabilizing MT attachments. It also regulates the recruitment of other components to the kinetochore; interestingly, these components include proteins such as Mad1 and Mad2, whose kinetochore accumulation patterns are different from those of the RanGAP1-RanBP2 complex. However, the RanGAP1-RanBP2 complex is not essential for Mad2-dependent checkpoint arrest. Spindle pole organization is disrupted in the absence of RanBP2, indicating a direct or indirect role of the RanGAP1-RanBP2 complex in additional aspects of spindle assembly and mitotic function.

Supplemental Data

Supplemental Data including Experimental Procedures and four figures are available at http://www.current-biology.com/cgi/content/full/14/7/611/DC1/.

Acknowledgments

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